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Activation of Autonomic Nerves and the Adrenal Medulla Contributes to Increased Glucagon Secretion During Moderate Insulin-Induced Hypoglycemia in Women

Peter J. Havel and Bo Ahren

Despite evidence that the autonomic nervous system (ANS) makes a significant contribution to increased glucagon secretion during insulin-induced hypoglycemia in several animal species, including a recent study in nonhuman primates, the role of the ANS in mediating this important counterregulatory response in humans remains controversial. Therefore, glucagon responses to insulin-induced hypoglycemia were examined in seven nondiabetic women (BMI, $28.0 \pm 2.0 \text{ kg/m}^2$) with and without the presence of the ganglionic nicotinic receptor antagonist trimethaphan. Trimethaphan impairs neurotransmission across parasympathetic and sympathetic autonomic ganglia and in the adrenal medulla and, therefore, markedly impairs autonomic activation during insulin-induced hypoglycemia. The studies were performed in random order at least 4 weeks apart. Trimethaphan was infused at a variable rate ($0.3\text{--}0.6 \text{ mg/min}$) to modestly lower blood pressure ($\sim 10 \text{ mmHg}$) without producing hypotension. Regular human insulin was infused ($0.28 \text{ pmol} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$) with a variable rate glucose infusion to lower the plasma glucose from 4.9 ± 0.2 to $2.6 \pm 0.2 \text{ mmol/l}$ in the control study and from 4.9 ± 0.2 to $2.5 \pm 0.2 \text{ mmol/l}$ in the trimethaphan study. Trimethaphan impaired parasympathetic and sympathoadrenal activation during insulin-induced hypoglycemia as assessed by 70% reductions of the plasma pancreatic polypeptide response and epinephrine response (both $P < 0.05$ vs. control study). Glucagon secretory responses during insulin-induced hypoglycemia were assessed as peak responses and as the area under the curve (AUC) above baseline values during insulin-induced hypoglycemia. Plasma glucagon increased in the control study from $44 \pm 5 \text{ ng/l}$ to a peak of $76 \pm 9 \text{ ng/l}$ ($\Delta = 32 \pm 8 \text{ ng/l}$; $P < 0.005$ vs. baseline) and in the trimethaphan study from 41 ± 3 to $50 \pm 7 \text{ ng/l}$ ($\Delta = 10 \pm 5 \text{ ng/l}$; $P < 0.02$ vs. control subjects). The glucagon response to insulin-induced hypoglycemia as assessed by the AUC was $948 \pm 272 \text{ ng} \cdot \text{l}^{-1} \cdot 45 \text{ min}^{-1}$ in the control study ($P < 0.01$ vs. baseline), but was reduced by 75% in

the trimethaphan study ($\text{AUC} = 203 \pm 94 \text{ ng} \cdot \text{l}^{-1} \cdot 45 \text{ min}^{-1}$; $P < 0.02$ vs. control subjects). Trimethaphan did not affect the glucagon response to arginine administration. These results demonstrate that the ANS mediates the majority of the glucagon response to insulin-induced hypoglycemia of 2.5 mmol/l in postmenopausal nondiabetic women. *Diabetes* 46:801–807, 1997

Increased glucagon secretion is a key element in the counterregulatory defense against hypoglycemia (1,2). However, the contribution of the autonomic nervous system (ANS) versus the direct islet effects of hypoglycemia per se in mediating this response have been difficult to separate. Hypoglycemia activates three different autonomic inputs to the pancreas: pancreatic parasympathetic nerves (3,4), the adrenal medullary hormone epinephrine (5), and pancreatic sympathetic nerves (6). Since each of these autonomic inputs is capable of stimulating glucagon secretion (7), we have hypothesized that the glucagon response to hypoglycemia is redundantly mediated such that all three autonomic inputs must be simultaneously blocked in order to demonstrate the autonomic contribution. Using approaches that impair all three inputs, an important autonomic contribution has been demonstrated in several animal species (7,8), including a recent study in nonhuman primates (9). However, the role of the ANS in mediating increased glucagon secretion during hypoglycemia in humans has not been established. Early human experiments addressing this question in which all of the autonomic inputs to the pancreas were not blocked (10) did not find an autonomic component, perhaps due to redundancy between the three inputs (11).

However, more recent studies using antagonists to block both classical cholinergic and adrenergic receptors also did not find an autonomic contribution in human subjects (12,13). These data, coupled with the observation that low glucose concentrations can stimulate glucagon secretion in vitro (14,15), suggest that the ANS may not contribute to the α -cell secretory response to hypoglycemia in humans. In contrast, there are some studies that have suggested an autonomic contribution in humans (8).

To address this controversy, moderate insulin-induced hypoglycemia of 2.5 mmol/l was induced, and glucagon secretory responses were assessed in seven healthy women on two occasions in random order with or without the infusion of the ganglionic blocking agent trimethaphan.

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ANS, autonomic nervous system; AUC, area under the curve; PP, pancreatic polypeptide.

Trimethaphan impairs neurotransmission across autonomic ganglia and in the adrenal medulla (16), reducing the overall autonomic response to hypoglycemia (9,17). Plasma pancreatic polypeptide (PP), an index of activation of pancreatic parasympathetic nerves (3,4), and plasma catecholamine responses, indexes of sympathoadrenal activation, were measured to assess the degree of autonomic activation in the control study and the efficacy of trimethaphan to reduce the autonomic activation. To determine if trimethaphan directly inhibits α -cell secretion, glucagon secretory responses to arginine administration were assessed in the presence and absence of trimethaphan.

RESEARCH DESIGN AND METHODS

Subjects. Seven healthy women were recruited from a large population screened for the prevalence of diabetes in Malmö, Sweden (18). All subjects in this population were born in 1931 (i.e., they were 64 years of age at the time of the study [mean \pm SD, 64 years and 3.0 ± 1.9 months at the time of the first experiment]). All subjects had normal glucose tolerance, as judged from a 75-g oral glucose tolerance test, according to the WHO criteria performed before entry the study. The BMI of the women was 28.0 ± 2.0 kg/m². All subjects were in good health and not taking any medication. Each subject underwent two separate experiments in random order (hyperinsulinemic hypoglycemic clamp \pm infusion of trimethaphan) with 4–6 weeks between studies. The effect of trimethaphan on glucagon secretory responses to arginine was assessed in six other healthy women with normal glucose tolerance (age, 58 ± 1 years; BMI, 25.9 ± 1.5 kg/m²). The study protocols were approved by the ethics committee of the Faculty of Medicine, Lund University, before the study. Written informed consent was obtained from all participants.

Experiments. Following an overnight fast, studies began at 0800. Indwelling catheters were inserted into antecubital veins of both arms. One arm was used for the infusion of glucose, insulin, and trimethaphan. The contralateral arm was used for intermittent blood sampling, and the catheter was kept patent with slow infusion of 0.9% saline.

Blood pressure was recorded regularly during the experiments. Two baseline samples for glucose, glucagon, PP, epinephrine, and norepinephrine were taken. Then, an intravenous infusion of saline or trimethaphan (Hoffman-LaRoche, Nutley, NJ) was started. The initial trimethaphan infusion rate was $0.3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ in a volume of 55 ml/h. After 10 min, the infusion rate was increased to $0.45 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ if systolic blood pressure had not dropped by 10 mmHg (6 subjects), and a further increase was made at 20 min to $0.6 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ if systolic blood pressure had not dropped by 10 mmHg (3 subjects). Trimethaphan was infused until min 90. At min 30, a primed constant infusion of insulin (Actrapid 100 U/ml, Novo Nordisk, Bagsvaerd, Denmark) with a constant infusion rate of $0.28 \text{ nmol} \cdot \text{m}^{-2} \text{ body surface area} \cdot \text{min}^{-1}$ was started. Blood glucose was determined every 5 min by the glucose dehydrogenase technique with a HemocueR (Hemocue AB, Ängelholm, Sweden) during the hyperinsulinemic hypoglycemic clamp. When blood glucose dropped to 3.0 mmol/l , a variable rate 20% glucose infusion was added, and the glucose infusion rate was adjusted manually until min 90 to maintain the blood glucose level close to 2.5 mmol/l . At min 90, the infusions of trimethaphan or saline and insulin were stopped, and the rate of glucose infusion was increased to restore euglycemia by min 120. Samples for analysis of plasma glucose, PP, epinephrine, norepinephrine, and glucagon were taken every 15 min during the infusions. Throughout each experiment, a senior physician (B.A.) was present at bedside to frequently monitor blood pressure and blood glucose and adjust the trimethaphan and glucose infusion rates to ensure that frank hypotension or hypoglycemia below the target threshold of 2.5 mmol/l did not occur.

The acute glucagon secretory response to arginine was measured in six other women on two occasions in random order either during infusion of saline or infusion of trimethaphan as described above. Two baseline samples were drawn; then 5 g of arginine HCl was administered intravenously, and blood samples were collected at 2, 3, 4, and 5 min.

Assays and data analysis. Blood samples for plasma analysis of glucose, PP, epinephrine and norepinephrine, and glucagon were immediately centrifuged at 5°C and serum frozen at -70°C until analyzed in duplicate. Plasma glucose concentrations were analyzed using the glucose oxidase method (19). PP was determined with double-antibody radioimmunoassay using rabbit anti-human PP antibodies. [¹²⁵I]-labeled human PP and human PP standard with reagents from Linco Research (St. Charles, MO) (20). Analysis of glucagon concentration was performed with double-antibody radioimmunoassay using guinea-pig anti-human glucagon antibodies specific for pancreatic glucagon.

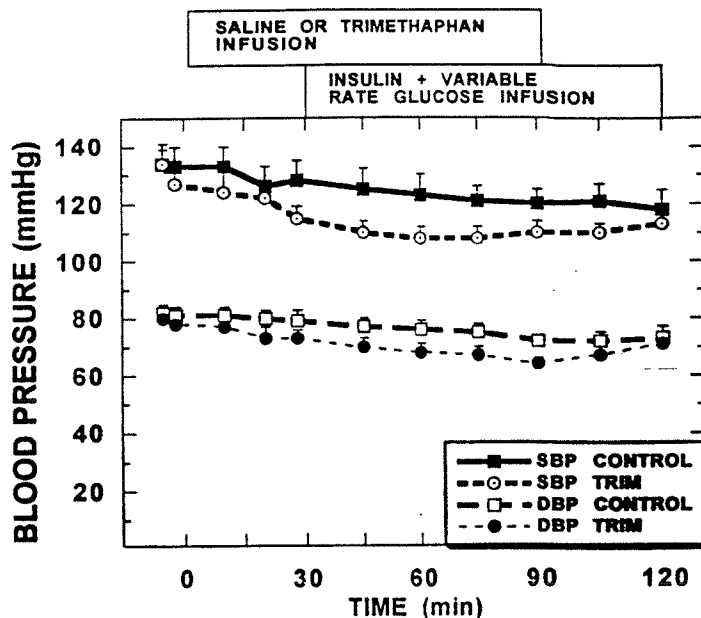


FIG. 1. Systolic (SBP) and diastolic (DBP) blood pressure before and during insulin plus variable-rate glucose infusion in seven women during saline infusion (CONTROL) and in the same women during ganglionic blockade with trimethaphan (TRIM).

[¹²⁵I]-glucagon as tracer, and glucagon standard (Linco Research). Plasma epinephrine and norepinephrine were determined by high-performance liquid chromatography as described (21,22). All concentrations were taken as the means of duplicate samples.

Data in the text, tables, and figures are presented as means \pm SE. Differences between the studies with saline versus ganglionic blockade with trimethaphan were performed with Wilcoxon's nonparametric signed-rank test for paired observations. Area under the curve (AUC) responses above baseline values between minutes 60–105 were calculated by the trapezoidal method. Glucagon responses were correlated with the PP, epinephrine, and norepinephrine responses, and the amount of glucose infused per kilogram of body weight in each study, by linear regression analysis.

RESULTS

Blood pressure. Baseline systolic blood pressure averaged 133 ± 7 mmHg and decreased modestly to 119 ± 5 mmHg during insulin-induced hypoglycemia in the control study ($P < 0.02$). Systolic blood pressure decreased from 131 ± 6 to 114 ± 4 mmHg during trimethaphan infusion ($P < 0.05$) and was lower (109 ± 4 mmHg) during hypoglycemia than in the control study ($P < 0.05$ vs. control subjects). Systolic blood pressure in the trimethaphan study recovered to control levels at min 120 after the trimethaphan infusion was discontinued at min 90 (Fig. 1). Diastolic blood pressure responses mirrored those described for systolic blood pressure (Fig. 1).

Plasma glucose. Baseline plasma glucose levels averaged 4.9 ± 0.2 mmol/l in both the saline and trimethaphan studies and were not affected by saline or trimethaphan infusion. Plasma glucose fell to and was maintained at similar levels during the insulin plus variable-rate glucose infusion during either saline (2.6 ± 0.2 mmol/l) or trimethaphan administration (2.5 ± 0.2 mmol/l). More glucose was infused in the trimethaphan study (32.5 ± 6.6 mg/kg) during the entire experiment, including the time periods when the plasma glucose concentration was maintained at 2.5 mmol/l and when the glucose infusion was increased to restore euglycemia, than in the control study (25.3 ± 5.5 mg/kg, $P < 0.05$ vs. trimethaphan). In both

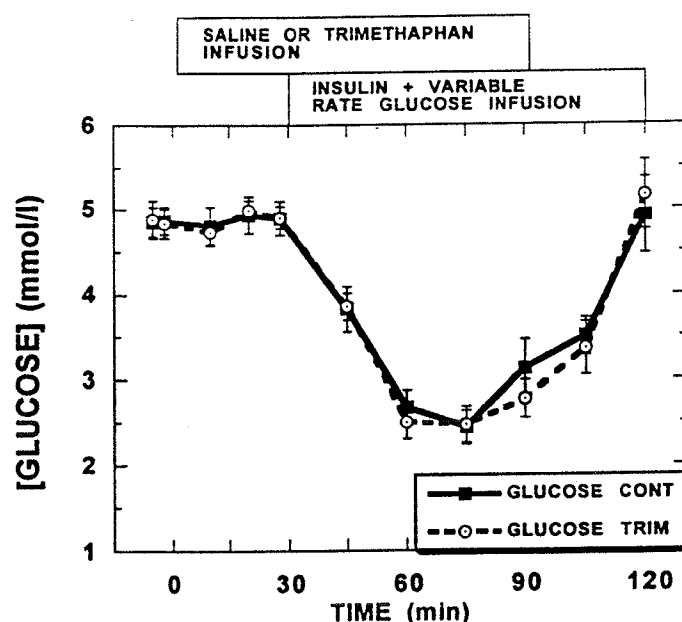


FIG. 2. Plasma glucose concentrations before and during insulin plus variable-rate glucose infusion in seven women during saline infusion (CONT) and in the same women during ganglionic blockade with trimethaphan (TRIM).

studies, increasing the glucose infusion rate normalized the plasma glucose concentration by 120 min (Fig. 2).

Plasma PP. Baseline plasma PP concentrations were similar before the saline or trimethaphan infusions. Plasma PP was lowered by 64 ± 26 pmol/l ($P < 0.025$) during trimethaphan infusion before the induction of hypoglycemia versus a tendency for PP to increase in the control experiment ($+53 \pm 33$ pmol/l, $P < 0.02$ vs. trimethaphan). Plasma PP concentrations increased markedly during hypoglycemia in the control study ($P < 0.005$). The increase of PP during hypoglycemia was attenuated by $71 \pm 16\%$ by ganglionic blockade with trimethaphan ($P < 0.0025$ vs. saline) (Table 1, Fig. 3). Plasma

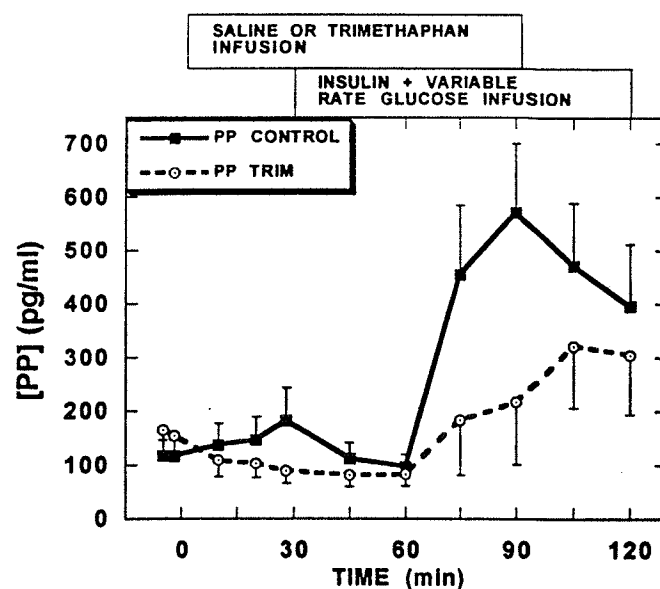


FIG. 3. Plasma PP concentrations before and during insulin plus variable-rate glucose infusion producing hypoglycemia of 2.5 mmol/l in seven women during saline infusion (CONTROL) and in the same women during ganglionic blockade with trimethaphan (TRIM).

PP tended to increase after the trimethaphan infusion was discontinued (i.e., between min 90 and 120).

Plasma catecholamines. Baseline plasma epinephrine was low in both studies. Epinephrine increased markedly during hypoglycemia in the control study ($P < 0.02$). The increase of epinephrine was attenuated ($\sim 70\%$) during ganglionic blockade ($P < 0.05$ vs. saline; Table 1, Fig. 4). Baseline plasma norepinephrine was similar in the saline and trimethaphan experiments. The plasma norepinephrine concentration doubled during hypoglycemia in the control study ($P < 0.02$). Plasma norepinephrine increased slightly, but significantly ($P < 0.02$) during hypoglycemia when trimethaphan was infused but the response was $< 20\%$ of the increase in the control

TABLE 1

Plasma hormone concentrations before and after trimethaphan or saline and during insulin-induced hypoglycemia, and AUC during insulin-induced hypoglycemia

| | Baseline (-10 to 0 min) | Trimethaphan/saline (20 to 30 min) | Insulin-induced hypoglycemia | |
|--------------------------------|----------------------------|---------------------------------------|------------------------------|--|
| | | | Peak (75 or 90 min) | AUC ($\text{ng} \cdot \text{l}^{-1} \cdot 45 \text{ min}^{-1}$) (60 to 105 min) |
| Plasma PP (pg/ml) | | | | |
| Saline | 118 ± 29 | 165 ± 52 | $571 \pm 130^*$ | $15,219 \pm 3,946^*$ |
| Trimethaphan | 160 ± 48 | $96 \pm 25^*$ | $218 \pm 116^{*+}$ | $5,254 \pm 3,398^{*+}$ |
| Plasma epinephrine (nmol/l) | | | | |
| Saline | 0.09 ± 0.02 | 0.06 ± 0.02 | $2.56 \pm 0.86^*$ | $83.1 \pm 29.9^*$ |
| Trimethaphan | 0.10 ± 0.03 | 0.05 ± 0.01 | $0.99 \pm 0.72^{*+}$ | $24.6 \pm 15.9^{*+}$ |
| Plasma norepinephrine (nmol/l) | | | | |
| Saline | 1.61 ± 0.21 | 1.54 ± 0.22 | $3.07 \pm 0.44^*$ | $64.8 \pm 8.9^*$ |
| Trimethaphan | 1.79 ± 0.27 | $1.42 \pm 0.16^*$ | $1.89 \pm 0.15^{*+}$ | $30.0 \pm 8.2^{*+}$ |
| Plasma glucagon (ng/l) | | | | |
| Saline | 44 ± 5 | 46 ± 6 | $76 \pm 9^*$ | $948 \pm 272^*$ |
| Trimethaphan | 41 ± 3 | 40 ± 3 | $50 \pm 7^{*+}$ | $203 \pm 94^{*+}$ |

Data are means \pm SE. $^*P < 0.05$ vs. baseline; $^+P < 0.05$ vs. saline.

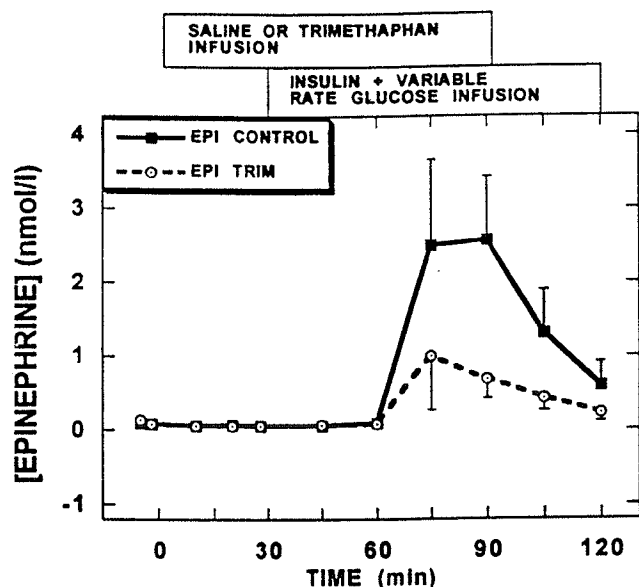


FIG. 4. Plasma epinephrine (EPI) concentrations before and during insulin plus variable-rate glucose infusion producing hypoglycemia of 2.5 mmol/l in seven women during saline infusion (CONTROL) and in the same women during ganglionic blockade with trimethaphan (TRIM).

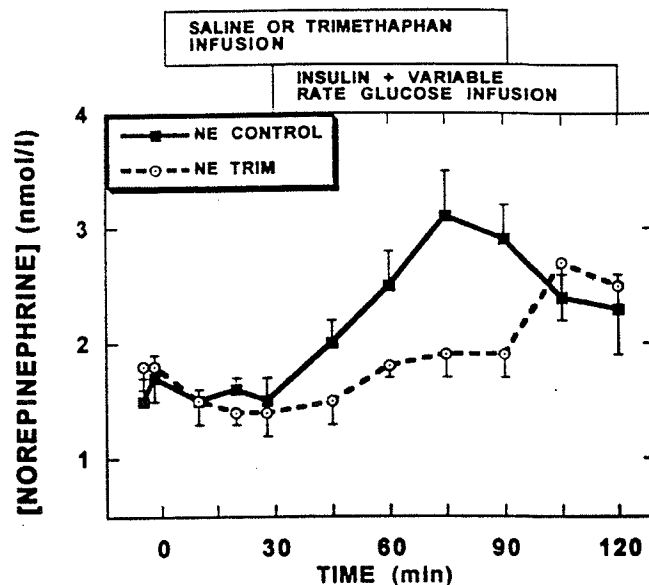


FIG. 5. Plasma norepinephrine (NE) concentrations before and during insulin plus variable-rate glucose infusion producing hypoglycemia of 2.5 mmol/l in seven women during saline infusion (CONTROL) and in the same women during ganglionic blockade with trimethaphan (TRIM).

study ($P < 0.02$ vs. saline). Similar to PP, plasma norepinephrine tended to increase after the trimethaphan infusion was discontinued (Table 1, Fig. 5).

Plasma glucagon. Baseline plasma glucagon was unchanged during saline or trimethaphan infusion. The increase of plasma glucagon during hypoglycemia ($P < 0.01$) was reduced by $72 \pm 17\%$ during hypoglycemia by ganglionic blockade with trimethaphan ($P < 0.005$ vs. control subjects; Table 1, Fig. 6). Overall, the percentage increase of plasma glucagon during hypoglycemia correlated with the percentage increase of plasma PP ($r = 0.54$, $P < 0.05$). The plasma glucagon responses to hypoglycemia, expressed as the integrated AUC between 60 and 105 min in the both control and trimethaphan studies, were positively correlated with the AUC responses for epinephrine ($r = 0.62$, $P < 0.02$), but not with the AUC responses for norepinephrine ($r = 0.03$). In addition, there was a significant inverse correlation between the amount of glucose infused per kilogram body weight and the glucagon AUC response to hypoglycemia ($r = -0.59$, $P = 0.026$; Fig. 7). Plasma glucagon responses to arginine administration were not different in the presence or absence of trimethaphan, peaking at $132 \pm 30\%$ ($P < 0.005$) above baseline during saline infusion and at $135 \pm 24\%$ ($P < 0.0025$) above baseline during trimethaphan (Fig. 8).

DISCUSSION

The aim of this study was to determine the contribution of the ANS to increased glucagon secretion during insulin-induced hypoglycemia in human subjects. A significant autonomic contribution has been previously found in several species (11,17,23–26), including a recent study in nonhuman primates (9). In contrast, the autonomic contribution in humans has been controversial. In the present study, pharmacological ganglionic blockade with trimethaphan was employed to impair the activation of the ANS during clamped hypo-

glycemia of 2.5 mmol/l. Trimethaphan was determined to be effective in reducing both parasympathetic and sympathoadrenal activation during hypoglycemia as assessed by diminution of the PP and plasma catecholamine responses to hypoglycemia, respectively. Ganglionic blockade produced a concomitant 75% impairment of the increase of plasma glucagon during hypoglycemia, suggesting an important autonomic component to the α -cell secretory response to hypoglycemia in nondiabetic human subjects. It is unlikely that the reduced glucagon response to hypoglycemia is due to a direct suppressive effect of trimethaphan on the α -cell, since we found that trimethaphan did not affect the glucagon response to arginine.

These findings conflict with those from a number of previous experiments in human subjects that did not find an autonomic contribution (27–30). Examination of the experimental design of those studies versus the present study reveals a significant difference that may explain the apparent discrepancy. In those studies, all of three autonomic inputs to the pancreas were not blocked, whereas in the present study, activation of at least two inputs (pancreatic parasympathetic nerves and the adrenal medulla) and most likely the third input (pancreatic sympathetic nerves) (6) was simultaneously impaired. Collectively, these previous data and our present results suggest that there may be redundancy between the autonomic inputs in the autonomic regulation of the glucagon response to hypoglycemia in humans, as has been previously described in rats (11).

Two other more recent studies in humans in which glucagon responses to hypoglycemia were assessed during muscarinic blockade with atropine in combination with α - and β -adrenergic blockade did not find a reduction of the glucagon response by these classical autonomic receptor antagonists (12,13). In contrast, one brief report utilizing ganglionic blockade (31) and two studies of patients with auto-

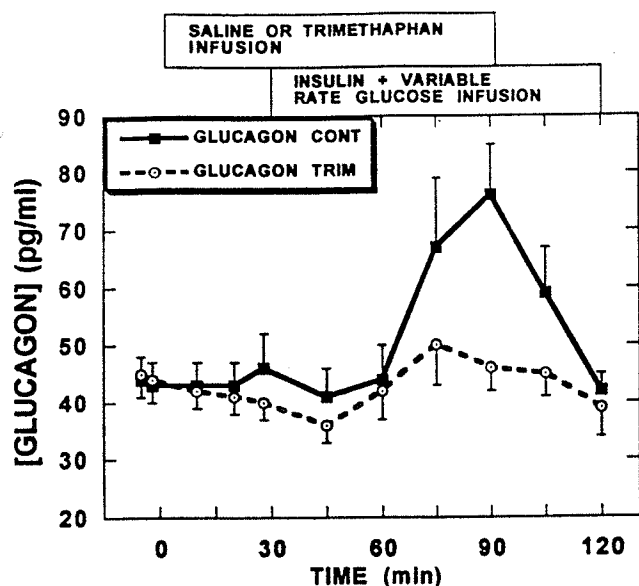


FIG. 6. Plasma immunoreactive glucagon concentrations before and during insulin plus variable-rate glucose infusion producing hypoglycemia of 2.5 mmol/l in seven women during saline infusion (CONT) and in the same women during ganglionic blockade with trimethaphan (TRIM).

normic disease (32,33) have suggested that combined parasympathetic and sympathoadrenal impairment can inhibit the glucagon response to hypoglycemia in humans. In addition, both ganglionic blockade and high-dose classical receptor blockade impair the glucagon response to hypoglycemia in rhesus monkeys (9). Together, these results suggest two possibilities. First, it is conceivable that the lower doses of antagonists employed in the human studies did not produce complete receptor blockade. In one study, similar doses of atropine were not sufficient to block cardiovascular parasympathetic reflexes (34). Although the degree of autonomic blockade produced by trimethaphan in the present study was not absolute, as assessed by significant albeit markedly reduced PP and catecholamine responses to hypoglycemia, it appears to be sufficient to significantly impair the glucagon response, whereas the doses of classical autonomic antagonists in previous studies (12,13) may not have sufficiently impaired the autonomic inputs to the pancreas. Second, there may be peptidergic component to the glucagon response to hypoglycemia in human subjects that is blocked at the level of the autonomic ganglia, but not by classical receptor antagonists. Several different peptidergic neurotransmitters have been identified in the pancreatic islets. However, a physiological role for these putative neuropeptides in regulating islet hormone secretion remains to be established (8). In any case, additional studies will be necessary to address the discrepancies between the effects of ganglionic blockade versus classical autonomic receptor blockade on hypoglycemia-induced glucagon secretion in humans.

In the present study, the magnitude of the glucagon response to hypoglycemia was significantly correlated with the magnitude of the PP and epinephrine responses, suggesting that the degree of parasympathetic and adrenal medullary activation are determinants of the glucagon response. The glucagon response was not correlated with the norepinephrine

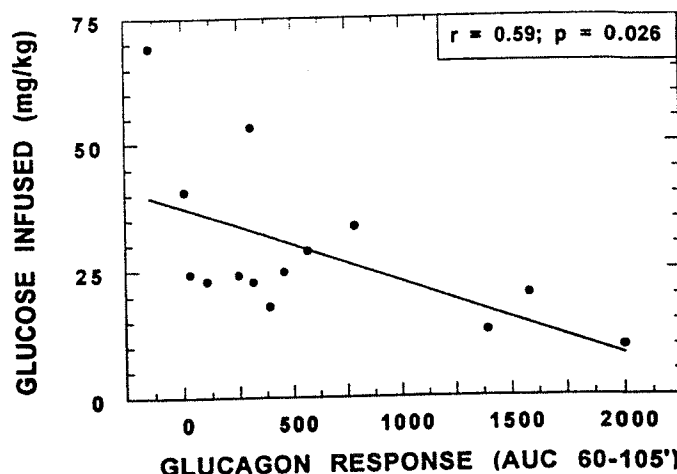


FIG. 7. Correlation between the amount of glucose infused per kilogram body weight and the AUC of the glucagon response to insulin-induced hypoglycemia in each study.

response. However, it should be noted that circulating norepinephrine is unlikely to be an important hormonal mediator of insulin or glucagon secretion (35,36), except during extreme stress. Rather, it is more likely to be norepinephrine released from sympathetic nerves innervating the pancreas, which can be dissociated from peripheral norepinephrine responses (37), that would stimulate glucagon secretion during hypoglycemia. Accordingly, pancreatic sympathetic nerves are activated during hypoglycemia (6) and contribute to hypoglycemia-induced glucagon secretion in dogs (38). Lastly, the inverse correlation between the glucagon response to hypoglycemia and the amount of glucose necessary to maintain the plasma glucose at 2.5 mmol/l underscores the important role of increased glucagon secretion in preventing moderate hypoglycemia from progressing to more severe life-threatening hypoglycemia.

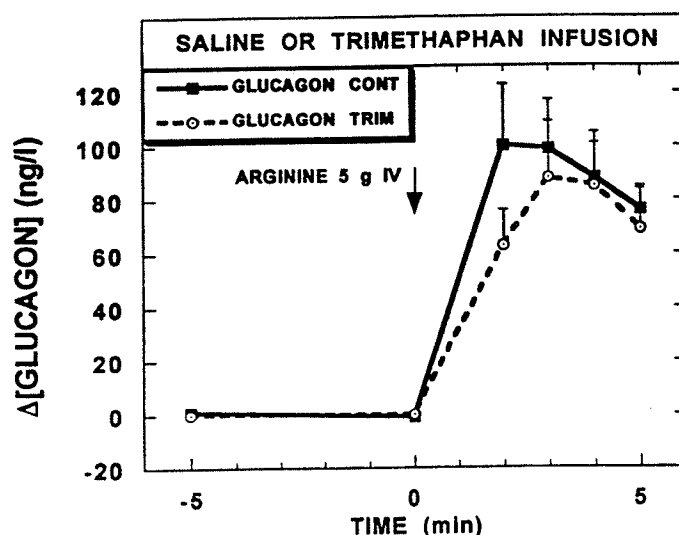


FIG. 8. The change of plasma glucagon above baseline values in response to intravenous administration of arginine (5 g) in six women during saline administration (CONT) and in the same six women during trimethaphan infusion (TRIM).

Despite the evidence for an autonomic contribution to the glucagon response to hypoglycemia in the present study, several caveats should be noted. First, although the glucagon response was attenuated, a significant increase of plasma glucagon persisted during ganglionic blockade. This could be explained by incomplete blockade as suggested by the incomplete suppression of the PP response. In this case, the autonomic contribution to glucagon response would exceed 75%. Alternatively, it is possible that other factors, including direct islet effects of low glucose concentrations (14,15), act to stimulate glucagon secretion during hypoglycemia. Second, this study was conducted in women, whereas most other studies have been performed in men or in both sexes. Since there are known gender differences in counterregulatory responses (39), the results of the present study should not be extended to males. Lastly, the present study was conducted in 64-year-old women who could potentially have a different autonomic component than younger subjects. However, since older individuals (67–84 years) have been shown to have reduced glucagon and epinephrine responses to hypoglycemia (40), it would seem more likely that the autonomic contribution would decrease rather than increase with age.

In summary, pharmacological ganglionic blockade reveals an important autonomic contribution to increased glucagon secretion during moderate insulin-induced hypoglycemia in postmenopausal nondiabetic women. These results suggest the possibility that defects of autonomic activation (e.g., autonomic neuropathy) or a decreased ability of the α -cell to respond to autonomic stimuli could contribute to the impaired glucagon response to hypoglycemia often observed in diabetes.

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